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Evidence for a Common Pattern of Peptidergic Innervation of Cnidocytes

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Abstract. Tentacles from representatives of all four classes of the phylum Cnidaria were examined using antibodies against the neuropeptides FMRFamide and RFamide to reveal the organization of neurons and nerve nets associated with cnidocytes. The tentacles of all species examined contained FMRFamide- or RFamide-immunoreactive neurons, in varying densities. In representatives from the Scyphozoa, Hydrozoa, and Cubozoa, the FMRFamide-immunoreactive neurons formed plexuses at the base of the cnidocyte assemblages; in anthozoans, the absence of discrete assemblies of cnidocytes precluded visual co-localization of cnidocytes and immunoreactive neurons. In all four classes, immunoreactive sensory cells connected these peptidergic nerve nets to the surface of the tentacle. These findings suggest that members of all four cnidarian classes share a common organizational pattern, and it is proposed that this peptidergic innervation may be involved in the chemosensory regulation of cnidocyte discharge.

Introduction

Cnidocytes, the sting cells of members of the phylum Cnidaria, are used for a variety of functions, including food capture, locomotion, intra- and interspecific aggression, and defense. Cnidocyte discharge is very tightly regulated, presumably to minimize what is likely to be the considerable energetic cost of replacing very complex cells that can only be used once. Early studies (Parker and Van Alstyne, 1932; Pantin, 1942) developed the concept that cnidocytes functioned as independent effectors that required both chemical and mechanical stimuli for discharge, and that single cnidocytes bore receptors for both stimulus modalities. While there is considerable evidence to suggest that single cnidocytes do indeed bear both chemoreceptors (Thurm *et al.*, 1998) and mechanoreceptors (Brinkmann *et al.*, 1995; Thurm *et al.*, 1998) and can discharge in the absence of neurons (Aerne *et al.*, 1991), it is now generally accepted that they are not completely independent effectors. Instead, their discharge is thought to be regulated by a variety of intrinsic factors (Thurm *et al.*, 1998), including the state of satiation of the animal (Sandberg *et al.*, 1971; Smith *et al.*, 1974).

A variety of ultrastructural (for review, see Westfall, 2004) and histological (Anderson *et al.*, 1992; Golz, 1994) studies have reported on the association between nerves and cnidocytes, and it is now clear that cnidocytes are innervated by both sensory neurons and interneurons (Westfall, 2004). Ultrastructural studies of the innervation of cnidocytes in anemones (Anthozoa) have revealed the presence of a variety of types of synaptic vesicles, including both dense- and light-cored vesicles, suggesting the presence of a variety of transmitters, including Antho-RFamide peptides (Westfall, 2004). Here we expand on this understanding of the role of the peptidergic innervation of cnidocytes by demonstrating, in representatives of the three other cnidarian classes (Hydrozoa, Scyphozoa, and Cubozoa), a common pattern of peptidergic innervation of cnidocytes. This consists of a network or basket of peptidergic neurons that surrounds the base of clustered cnidocytes, together with a finite number of peptidergic sensory neurons that send processes to the surface of the tentacle.

Materials and Methods

Specimens of *Chrysaora quinquecirrha* (Desor, 1848) (Class Scyphozoa), *Physalia physalis* (Linnaeus, 1758), *Porpita porpita* (Linnaeus, 1758) *Cladonema* (sp.) (Class

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Hydrozoa), *Chiropsalmus* (sp). (Class Cubozoa), and *Bunodosoma cavernata* (Bose, 1802) (Class Anthozoa) were collected in the vicinity of the Whitney Laboratory and maintained in running seawater at ambient temperatures. Pieces of tentacle or, in the case of *Cladonema,* small portions of colonies, were anesthetized in isotonic (0.37 *M*) $MgCl_2$ mixed 1:1 with seawater. Relaxed tentacles were then lightly stretched and pinned, using cactus spines (*Opuntia* sp.), onto a layer of Sylgard at the base of a petri dish. Individual polyps were excised from *Cladonema* colonies.

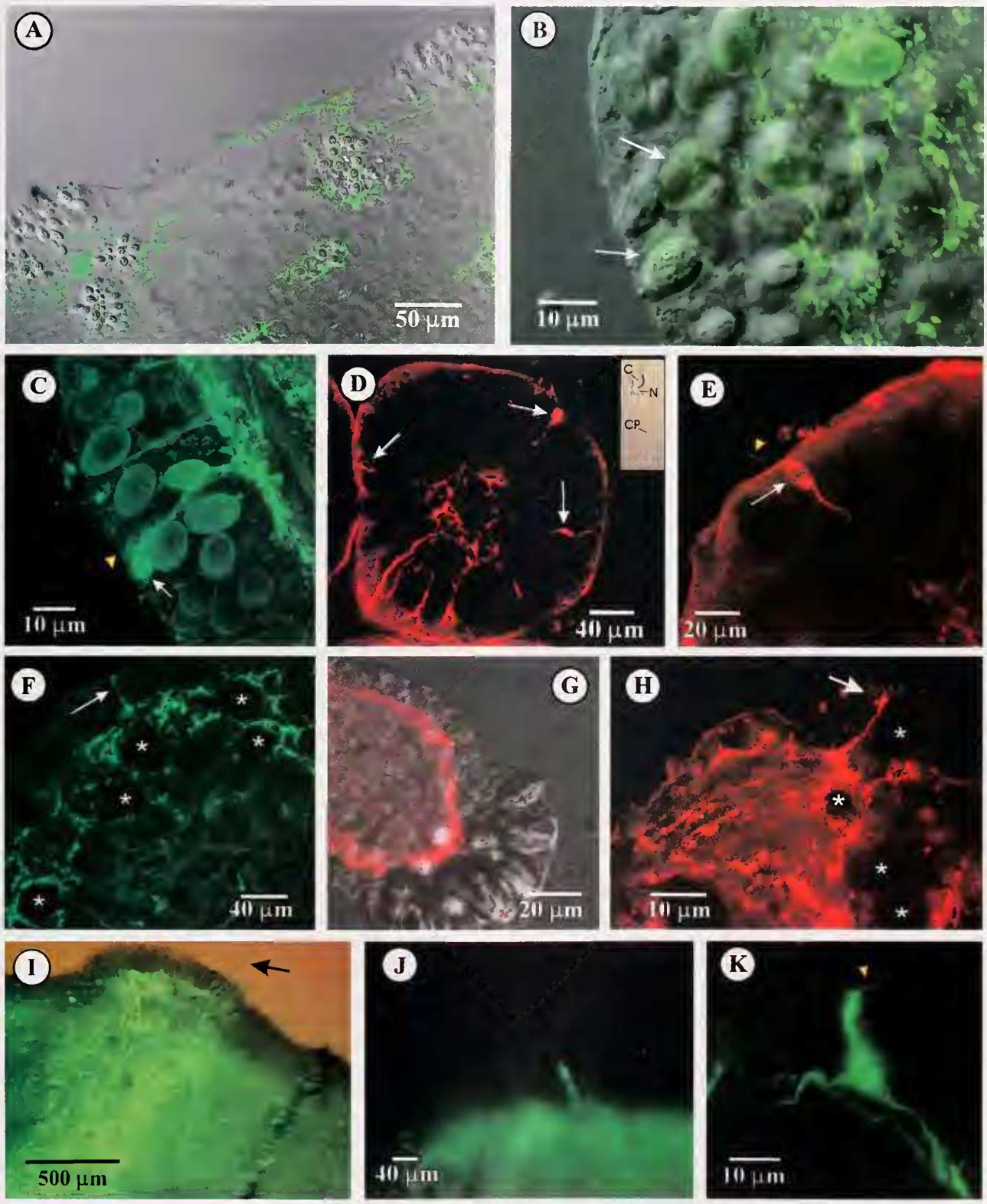
Excised tissue was then prepared for immunocytochemistry as described by Grimmelikhuijzen (1985). Briefly, tissues were fixed in 4% phosphate-buffered paraformaldehyde (pH 7.0) overnight at 4 °C, and rinsed (6 × 1 h) in phosphate-buffered saline containing 0.25% Triton X-100 (PBS-T). Samples were then incubated overnight at 4 °C in the presence of either an anti-FMRFamide antibody (Diasorin, Inc.) or an anti-RFamide antibody (146III provided by C. J. P. Grimmelikhuijzen, Univ. of Copenhagen) diluted 1:250 in PBS-T supplemented with 0.25% goat serum (PBS-T-G). After rinsing (4 × 1 h) with PBS-T, the samples were incubated overnight at 4 °C in secondary goat-anti-rabbit antibody conjugated to either FITC (Boehringer Mannheim) or Cy3 (Jackson Immunoresearch), diluted 1:150 with PBS-T-G. After a final round of rinsing, the samples were transferred to a drop of 90% glycerol in PBS containing 1 mg/ml o-phenylenediamine HCl on a microscope slide. The edges of the coverslip were then sealed with clear nail polish. Control samples were prepared in exactly the same manner, except that the secondary antibody was omitted. Samples were examined with either a Leica DMIRBE fluorescence microscope or a Leica laser scanning confocal microscope.

Results

In all the species examined, FMRFa-like immunostaining was present in the tentacles. In *Chrysaora, Cladonema, Physalia,* and *Porpita,* the staining was particularly dense with the anti-FMRFa antibody. In individual tentacles, immunoreactivity typically appeared as longitudinal arrays of neurons connected by transverse processes. In *Bunodosoma,* the anti-RFamide antibody staining was far more effective than anti-FMRFamide, and revealed a dense plexus of multipolar neurons throughout the ectoderm of the tentacles.

Equally common in all but *Aiptasia* were assemblies of immunoreactive neurons associated with cnidocytes. This organization was best illustrated in tentacles of *Chrysaora* (Fig. 1A), where cnidocytes clusters are more widely scattered than in the other species. In *Chrysaora,* immunoreactive nerve nets were associated with clusters of as few as

Figure 1. (A) Combined confocal fluorescence and Normarski image of part of a tentacle from *Chrysaora.* Note the green FMRFa-like immunoreactive nerve nets that are associated with each cluster of cnidocytes. (B) Combined laser scanning confocal/Normarski image of part of a tentacle from *Chrysaora.* Processes from the dense immunoreactive plexus at the base of the cnidocytes envelop (arrows) individual cnidocytes. Some cnidocysts display autofluorescence. (C) Laser scanning confocal image of a tentacle from *Chrysaora.* A presumed sensory neuron bearing a fine sensory process that projects into the external environment (yellow arrowhead) sends an axon to the dense immunoreactive plexus at the base of the cnidocytes. Note that several cnidocysts display autofluorescence. (D) Confocal image of a cnidosac from *Porpita.* Immunoreactive neurons (here stained red with Cy3) are present in the center of each sphere and connect the central plexus to the tentacle. Presumed sensory cells, with cell bodies at the surface of the cnidosacs and axons that connect to the central core of immunoreactivity, are evident (white arrows). Inset: Normarski photomicrograph of a single isolated cnidocyte from *Porpita* (same scale) showing the overall length of the cell. C, cnidocyst; CP, cytoplasmic processes; N, nucleus. (E) Higher magnification confocal image of the soma of a sensory cell (white arrow) in *Porpita* and its ciliary process (yellow arrowhead). (F) Confocal image of part of a cnidosac from *Physalia.* The cysts of the cnidocytes appear as unstained circles (asterisks). Note the presence of immunoreactive processes surrounding the cnidocytes, and a single cell that projects to the surface of the tentacle (arrow). (G) Combined laser-scanning confocal and Normarski image of the tip of a capitate tentacle of the hydroid *Cladonema.* Immunoreactivity is present in the ectoderm along the entire length of the tentacle, but is concentrated at the base of the cnidocytes. (H) Laser scanning confocal micrograph of the tip of a capitate tentacle of *Cladonema,* showing the presence of a dense immunoreactive plexus at the base of the cnidocytes, and a single immunoreactive process that extends to the surface of the tentacle (arrow). The cysts of the cnidocytes appear as unstained circles (asterisks). (I) A combined bright field/fluorescence image of part of a tentacle from *Chiropsalmus.* The cnidocytes are found in regularly spaced girdles that encircle the tentacles. Each ring of cnidocytes is associated with a dense array of immunoreactive (green-FITC) processes. At the edge of the tentacle it can be seen that these processes are located at the basal end of the cnidocytes. Threads from discharged cnidocysts (black arrow) show the location of the cnidocytes. (J) Fluorescence micrograph of part of a tentacle from *Chiropsalmus* showing a single immunoreactive cell (arrow) that projects to the surface of the tentacle, through the cnidocytes. (K) A laser scanning confocal image of part of a tentacle from the sea anemone *Bunodosoma,* showing a single immunoreactive sensory cell that projects to the surface of the tentacle. A single cilium from this cell (yellow arrowhead) projects into the mucus layer. This cell spans the width of the ectoderm of the tentacle, and connects with an immunoreactive nerve net located at the ectoderm-mesoglea interface.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two or three cnidocytes as well as with the larger assemblies of cnidocytes. These assemblages of immunoreactive neurons had the appearance of baskets composed of immunoreactive neurons 1–2 μm in diameter. At higher power (Fig. 1B), the immunoreactive neurons in *Chrysaora* could be seen to be located primarily at the base of the cnidocytes. However, individual processes emerged from this dense plexus and partially enveloped individual cnidocytes (Fig. 1B).

Also evident in *Chrysaora* were individual immunoreactive neurons whose somata were located at the surface of tentacles and extended axons to the immunoreactive plexus at the base of the cnidocytes (Fig. 1C). A single fine process could occasionally be seen to emerge from the cell soma into the external medium (Fig. 1C). These cells had the typical appearance of sensory cells.

In the two siphonophore species examined, *Porpita* and *Physalia*, the tentacles bear large assemblies of cnidocytes, called cnidosacs, that appear as a line of hemispheres or spheres arranged along one edge of each tentacle. In a well-relaxed tentacle, this organization gives the appearance of a string of small blue beads. In *Porpita*, the cnidosacs almost form self-contained spheres that are attached to the tentacle through a small stalk (Fig. 1D). The central core of each cnidosac was inevitably filled with large numbers of immunoreactive processes that were connected to the shaft of the tentacle by additional immunoreactive neurons. Cnidocytes from *Porpita* are remarkably long, and much of their length is taken up by a long (80 μm), narrow, basally directed cytoplasmic projection (Fig. 1D, inset). Thus, although the immunoreactivity in *Porpita* was located in the central core of the cnidosac, its location was still ectodermal, around the base of the long cytoplasmic projections.

Several sensory neurons were observed to extend to the surface of each cnidosac (Fig. 1D, E). These cells had large ovoid somata (Fig. 1E), which were typically located near the surface of the tentacle; many had a single fine process that extended into the extracellular medium (Fig. 1E). Occasionally, cells that projected to the surface of the cnidosac had more centrally located somata (Fig. 1D). The cnidosacs in *Porpita* were small enough to be fully reconstructed from confocal optical slices and provided a measure of the overall density of sensory neurons. A typical cnidosac contained up to 6–7 sensory neurons.

In *Physalia*, the cnidosacs are more hemispherical and the ectoderm thinner than *Porpita*. When cnidosacs were viewed from the surface, a dense plexus of immunoreactivity surrounding the dark cores of the cnidocytes was apparent (Fig. 1F). When viewed tangentially, it could be seen that the immunoreactivity was largely restricted to the base of the ectoderm of the cnidosac, around the base of the cnidocytes (Fig. 1F), but that occasional neurons emerged from the plexus and extended to the surface of the tentacle (Fig. 1F).

In the case of the hydroid *Cladonema*, the FMRFa-like innervation of the tentacle was particularly evident. Immunoreactive processes located in what appeared to be the base of the ectoderm covered the entire tentacle (Fig. 1G). This nerve net was particularly dense, however, at the base of the capitate portion of the tentacle, immediately under the cluster of cnidocytes (Fig. 1G, H). This organization was very reminiscent of that in the two siphonophores and of that reported for another hydroid, *Coryne* (Golz, 1994). Once again, single neurons occasionally emerged from this plexus and extended through the cnidocytes to the surface of the tentacle (Fig. 1H).

In the cubomedusa *Chiropsalmus*, the bulk of tentacular cnidocytes exist in regular bands that encircle the entire tentacle (Fig. 1I). Once again, these cnidocyte bands were associated with a dense plexus of immunoreactive neurons that was located in the ectoderm of the tentacle, at the base of the cnidocytes (Fig. 1I). Single immunoreactive cells could be seen to emerge from this plexus to the surface (Fig. 1J).

The pattern of immunoreactivity in the anthozoan *Bunodosoma* differed markedly from that of the other species examined. As noted above, while the anti-FMRFamide antibody was only partially effective, the anti-RFamide antibody revealed a dense network of multipolar neurons located at the base of the ectoderm. When the edges of tentacles were viewed tangentially, single ciliated sensory cells were evident (Fig. 1K) and processes from these merged with the immunoreactive nerve net. The cilia of these sensory cells emerged from the surface of the tentacle, but seemed to extend no further than the mucus layer on the surface.

Discussion

Intracellular recordings from cnidocytes in the tentacles of *Physalia* (Purcell and Anderson, 1995) during applications of aqueous extracts of fish mucus are characterized by bursts of small depolarizing potentials. Similar activity is triggered in cnidocytes in the capitate tentacles of *Cladonema* by applications of an aqueous extract of *Artemia* (Price and Anderson, unpubl. data). In both cases, the activity is absent in tentacles that are bathed in a high-Mg^{2+}, low-Ca^{2+} seawater, implying that the depolarizing events are synaptic potentials. The fact that these events occur synchronously in closely apposed or adjacent cnidocytes (Purcell and Anderson, 1995) suggests that clustered cnidocytes receive a common synaptic input that is triggered by chemosensory stimulation of the tentacle.

The results presented here indicate that cnidocytes in both these species, together with representatives from at least two other cnidarian classes (Scyphozoa, Cubozoa) are innervated by neurons that are immunoreactive to antibodies against the peptide FMRFamide. Irrespective of the organi-

zation of the cnidocytes, be they in small planar clumps (*Chrysaora*), broad circumtentacular bands (*Chiropsalmus*), single capitate bulbs (*Cladonema*), or the linear arrangement of nearly cnidosacs found in *Physalia* and *Porpita*, a dense plexus of FMRFamide IR neurons is present around the base of the cnidocytes. The basal location of this plexus is best exemplified by *Porpita*, where the long cytoplasmic extension at the basal end of each cnidocyte creates a considerable separation between the cyst and the FMRFamide IR neurons. The basal location of this plexus is consistent with the site of neuro-cnidocyte synapses reported by other investigators (Holtmann and Thurm, 2001; Westfall, 2004), although there is evidence—in *Chrysaora* at least—that FMRFamide IR processes extend partway up the length of some cnidocytes, presumably forming synapses around the midpoint of the cell.

The sea anemone *Bunodosoma* may appear to be the exception to this pattern of peptidergic innervation. While the tentacles did contain a dense plexus of FMRFamide and RFamide immunoreactive neurons, it was not possible to discern any obvious association between these neurons and cnidocytes in the tentacles. EM studies of cnidocytes in the anemone *Aiptasia* (Westfall, 2004), on the other hand, have revealed the presence of RFamide immunoreactive dense-cored vesicles at neuro-cnidocyte synapses, suggesting that cnidocytes in anemones are indeed innervated by peptidergic neurons. The lack of a clear association between the immunoreactive nerve nets and cnidocytes may, therefore, simply be a consequence of the anemone's anatomy. In *Bunodosoma* and other anemone species, the ectoderm of the tentacles is filled, almost uniformly, with cnidocytes and, unlike the representatives of the other classes examined, there are no discontinuities in cnidocyte coverage that could be mapped to interruptions or breaks in the underlying peptidergic nerve net. Thus, the presence of a peptidergic nerve net over the entire tentacle could merely be a reflection of the uniform distribution of cnidocytes in the tentacles.

A consistent feature of the FMRFamide IR plexus in all species examined was the presence of what appear to be peptidergic sensory neurons that emerge from the plexus. Their classification as sensory neurons is based on the peripheral location of the cell body and the presence, in many of them, of a very fine process that extends into the external medium, criteria that are consistent with those used by other investigators (Saripalli and Westfall, 1996). Cells in the capitate tentacles of the hydroid *Coryne* and classified as sensory cells (Holtmann and Thurm, 2001) contain dense cored vesicles that are consistent with their being peptidergic. Although the sensory cells described here are relatively rare (a single 200-μm-diameter cnidosac in *Porpita* contains only 6–7), the average density is on the order of one sensory process per 8,000–10,000 μm^2 of cnidocyte-rich tentacle surface. However, because those sensory cells are afferent to a dense nerve net, or plexus, the result is that this organization has the potential to directly or indirectly activate a great many cnidocytes. It is not clear whether all cnidocytes in a cluster receive synaptic input directly. It is possible that only a finite number of cnidocytes are innervated but that the afferent input is conveyed to other cnidocytes through signaling systems such as nitric oxide (Salleo *et al.*, 1996; Colasanti *et al.*, 1997; Moroz *et al.*, 2004) or by way of gap junctions, which are very abundant in hydrozoans (Josephson and Schwab, 1979; Spencer, 1981) and may also be present in anthozoans (Germain and Anctil, 1996; Mire *et al.*, 2000). However, while agents that uncouple gap junctions affect the responses of hydrozoan (Price and Anderson, unpubl. data) and anthozoan (Mire *et al.*, 2000) cnidocytes, cnidocytes in the hydroid *Stauridiosarsia* (Brinkmann *et al.*, 1996) are not dye-coupled to one another or to adjacent cells, making the possible role of gap junctions equivocal.

It is not altogether surprising that these cnidocyte-specific plexuses were revealed using antibodies to FMRFamide. RFamide peptides are exceedingly common in the Cnidaria (for review, see Grimmelikhuijzen *et al.*, 2002). They have been isolated from representatives of all classes, with the exception of the Cubozoa where peptide studies have not yet been conducted; and they have been shown to be functionally important for development (Katsukura *et al.*, 2003) and physiological activity (McFarlane *et al.*, 1987, 1991). However, cnidarians do not possess FMRFamide *per se*, but rather a variety of other peptides that terminate in the sequence RFamide peptides. The fact that FMRFamide antibodies were only minimally effective in *Bunodosoma* while RFamide antibodies were effective may reflect this point, and suggests that the use of the antiRFamide antibody with the other species might reveal more details of the peptidergic innervation of the cnidocytes.

It must be stressed, however, that the presence of an RFamide-like innervation of cnidocytes does not preclude the involvement of other neurotransmitter pathways. While the evidence for other types of neurotransmitter in the phylum Cnidaria is not as conclusive as for peptides, evidence for other transmitters is growing (for review, see Anderson, 2004). In addition, some evidence (Kass-Simon and Scappaticci, 2004) suggests that glutamatergic pathways may also be involved in the regulation of *Hydra* cnidocytes, and that dopamine is involved in modulating the discharge of cnidocytes from *Corynidae* (Thurm *et al.*, 1998). Furthermore, the finding that the threshold for cnidocyte discharge is raised in satiated animals (Sandberg *et al.*, 1971; Smith *et al.*, 1974) suggests that regulatory pathways must be present—ones that somehow inhibit or down-regulate cnidocyte discharge.

As noted earlier, application of an extract of fish mucus to *Physalia* tentacles triggers bursts of electrical activity that can be recorded as synaptic events in single cnidocytes

(Purcell and Anderson, 1995). That synaptic input does not, however, evoke discharge of the impaled cnidocytes, or any adjacent ones. This suggests, therefore, that the synaptic activity may serve to somehow prime the cnidocyte in preparation for the subsequent mechanical stimulus that would indicate physical contact has been made with the source of the odorant. Given the organization of the cnidocyte-associated peptidergic nerve nets presented here and the presence of what appear to be peptidergic afferent sensory neurons, it is tempting to speculate that the synaptic activity that can be recorded from the cnidocytes of *Physalia* (Purcell and Anderson, 1995) and *Cladonema* (Price and Anderson, unpubl. data) arises, directly or indirectly, from these peptidergic nerve nets. While there is no direct physiological or pharmacological evidence that peptides are involved in the cnidocyte response, there is no evidence that peptides are not involved. Furthermore, electron microscopic immunocytochemistry has revealed the presence of RFamide peptides in the nerve terminals of neurocnidocyte synapses in anemones (Westfall, 2004). Thus, given their prevalent role in many aspects of cnidarian biology and their clear association with cnidocytes in all cnidarian classes, it would be surprising if neuropeptides were not actively involved in some aspect of the regulation of cnidocyte discharge.

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